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Fluid Accumulation in Infant Mice Caused by *Vibrio hollisae* and Its Extracellular Enterotoxin

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***Vibrio hollisae*, a halophilic bacterium isolated from patients with diarrhea, was examined for virulence factor production. Intragastric administration of 2×10^7 CFU per mouse elicited fluid accumulation which peaked at ca. 6 h postchallenge in infant mice. An enterotoxin which elongated Chinese hamster ovary (CHO) cells was detected in extracts of infected-mouse intestines and in culture fluids from various growth media. The yield of the enterotoxin was maximal beginning at the onset of the stationary phase of growth in heart infusion broth supplemented with 0.5% NaCl. A concentrated preparation obtained by ammonium sulfate precipitation of culture supernatant fluids induced intestinal fluid accumulation which peaked at 2 h postchallenge in infant mice. The abilities of the enterotoxin preparation to elongate CHO cells and to elicit fluid accumulation in infant mice were inseparable by gel filtration, isoelectric focusing, and hydrophobic interaction chromatography. The enterotoxin has a molecular weight of ca. 33,000 by gel filtration and an isoelectric point of ca. 4 and is sensitive to heat.**

Vibrio hollisae, previously designated by the Centers for Disease Control, Atlanta, Ga., as group EF-13, is a halophilic bacterium which was isolated from patients with diarrhea and abdominal pains (15). In most cases, illness was associated with the consumption of raw seafood. Of the 16 strains originally identified as *V. hollisae*, 14 were from stool samples (9), and the clinical characteristics of the disease resembled those seen in cases of gastroenteritis caused by non-O1 *Vibrio cholerae* (11, 14). *V. hollisae* possesses sequences that have homology with the gene encoding the thermostable direct hemolysin of *Vibrio parahaemolyticus* (16), produces a hemolysin which is lytic for human and rabbit erythrocytes, and is antigenically similar to the thermostable direct hemolysin (M. Nishibuchi and J. B. Kaper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B90, p. 33). The lack of information regarding the elaboration of any other virulence factor(s) prompted us to investigate the possibility that *V. hollisae* produces a toxin which is important in pathogenesis of the enteric disease caused by the organism. In this paper, we report that *V. hollisae* elicits fluid accumulation in infant mice, and we describe the production and partial characterization of an extracellular enterotoxin which elongates Chinese hamster ovary (CHO) cells and causes fluid accumulation in infant mice.

MATERIALS AND METHODS

Bacterium and seed culture preparation. *V. hollisae* ATCC 33564 (CDC 0075-80) was obtained from the Centers for Disease Control. Frozen specimens of the organism were prepared by first growing the bacteria with shaking at 30°C to the mid-logarithmic phase of growth in heart infusion broth (Difco Laboratories, Detroit, Mich.) containing additional 0.5% NaCl (modified heart infusion broth) and then freezing the portions (1 ml) of the culture diluted with sterile bovine serum (0.5 ml) at −70°C. Seed cultures were prepared by spreading a Columbia agar plate (Difco) containing additional 0.5% NaCl for confluent growth with 3 loopsful of the frozen culture. After incubation for ca. 16 h at 30°C, the surface growth on the Columbia agar was collected with modified heart infusion broth (5 ml per petri culture), and the suspension was adjusted to an optical density at 650 nm (OD_{650}) of ca. 7.5 (ca. 6×10^9 CFU/ml).

Activity against CHO cells. The ability of the enterotoxin to cause elongation of CHO cells was estimated by a modification of the method described by Guerrant et al. (8). Stock cultures of CHO cells were grown at 37°C in a humidified atmosphere containing 5% CO_2, in Eagle minimum essential medium containing Hanks base salts (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO), 10% tryptose phosphate broth (Difco), 2 mM L-glutamine (GIBCO), 3% sodium bicarbonate, penicillin (50 IU/ml), and streptomycin (50 μg/ml). The cells were harvested with 0.05% EDTA–0.25% trypsin in Dulbecco phosphate-buffered saline (PBS), and portions of washed-cell suspensions (ca. 1,000 cells) in 0.1 ml of supplemented Eagle minimum essential medium containing 1% fetal bovine serum and without tryptose phosphate broth were added to wells of 96-well microtiter assay plates (Corning Glass Works, Corning, N.Y.). Serial twofold dilutions of the preparations to be tested were made in supplemented Eagle minimum essential medium without fetal bovine serum and tryptose phosphate broth, and 10 μl of each dilution was added to separate microtiter wells. The cells were examined for elongation after incubation in 5% CO_2 for ca. 18 h at 37°C. One CHO unit is defined as the reciprocal of the dilution that causes elongation of >50% of the cells.

Infant mouse assay. The ability of the *V. hollisae* cells and the enterotoxin to cause intestinal fluid accumulation was determined in infant mice. Pregnant (17 to 18 days) CFW mice were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, N.C.). Infant mice weighing ca. 2.5 to 4.0 g (5 to 6 days old) were starved for ca. 10 h at 30°C before being fed viable bacteria or the partially purified enterotoxin

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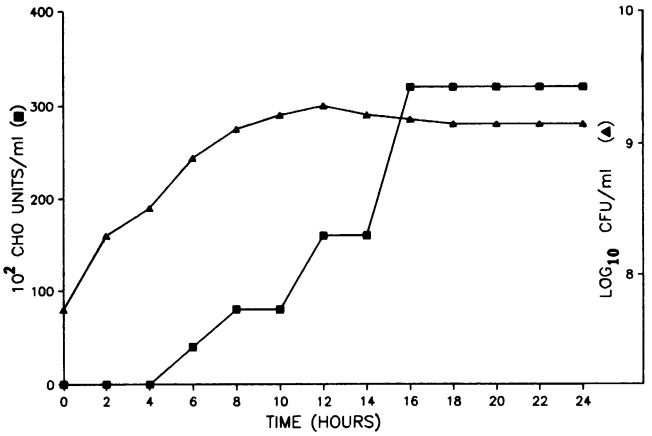

FIG. 1. Growth and enterotoxin production by *V. hollisae* ATCC 33564. A 2-liter benchtop fermentor containing 1,490 ml of modified heart infusion broth was inoculated with 10 ml of a seed culture suspension (OD_{650}, 75), and the culture was incubated at 25°C, infused with 0.75 liters of air per min, and agitated at an impeller speed of 250 rpm. Growth was determined by plate counts, and the culture supernatant fluids were obtained by centrifugation and assayed for activity against CHO cells.

preparations. The bacteria were grown for the infection studies by inoculating modified heart infusion broth (100 ml in 500-ml baffled flasks) with a loopful of culture from a Columbia agar plate and then incubating the medium for 12 h at 30°C with agitation at 250 rpm. The bacteria were collected by centrifugation and washed twice with 20 ml of PBS (0.02 M Na_2HPO_4–0.15 M NaCl, pH 7.4) before being suspended in 10 ml of the same buffer. The anorectal canals of mice were sealed with cyanoacrylate (Elmer's Wonder Bond; Borden, Inc., Columbus, Ohio), and a 0.25-ml glass tuberculin syringe fitted with a 23-gauge needle tipped with intramedic polyethylene tubing was used to inoculate the mice intragastrically with 50 μl of the sample. After the desired time interval, mice were sacrificed by cervical dislocation, and the fluid accumulation (FA) ratio was determined for each mouse as described by Baselski et al. (2). The FA ratio was expressed as 1,000 times the ratio of the weight of the stomach plus intestine to the remaining body weight. A total of 117 mice in a control group which received 50 μl of PBS and were sacrificed at different times showed a mean FA ratio ± standard deviation of 57.3 ± 3.7. Based on this value, FA ratios above 65 were considered positive.

The peroral median lethal dose of *V. hollisae* was determined with infant mice. *V. hollisae* was grown, cells were washed, and infant mice were inoculated per os as described above. Mice were observed for 24 h postchallenge, and the median lethal dose was calculated by the method of Reed and Muench (18).

The enterotoxin preparations were tested for protease activity against azocasein as previously described (12) and for cytolytic activity against mouse, sheep, rabbit, chicken, and human erythrocytes as described by Bernheimer and Schwartz (3). Protein in the partially purified enterotoxin preparation was estimated by the method described by Bradford (4), with bovine plasma albumin as the standard. The standard and the assay reagents were obtained from Bio-Rad Laboratories, Inc. (Richmond, Calif.).

Enterotoxin production. The ability of *V. hollisae* to produce enterotoxin in heart infusion broth containing different amounts of sodium chloride and in different media was examined by inoculating 5 ml of medium in 50-ml flasks with 30 μl of the seed culture suspension (OD_{650}, 0.25; ca. 2×10^8 CFU) and assaying the culture supernatant fluids after the cultures were incubated for 24 h at 30°C on a shaker operating at 250 cycles per min. Enterotoxin production in selected basal media was further examined by the procedure described above except that 100 ml of medium in a 500-ml baffled flask was inoculated with ca. 660 μl of the seed culture suspension (OD_{650}, 5.0; ca. 4×10^9 CFU). The media tested include 2% Bacto-tryptone, 2% Bacto-peptone, 2% Proteose peptone, Columbia broth, tryptose phosphate broth, marine broth, heart infusion broth, Casamino Acids, and synthetic broth AOAC (all from Difco), and Trypticase soy broth and brain heart infusion (both from BBL Microbiology Systems, Cockeysville, Md.).

Preparation of partially purified enterotoxin. Unless otherwise noted, all steps were at 4°C. A 10-ml portion of the seed culture suspension (OD_{650}, ca. 75) was added to 1,490 ml of modified heart infusion broth contained in a 2-liter benchtop fermentor (Multigen; New Brunswick Scientific Co., Inc., Edison, N.J.). Polypropylene glycol P-2000 (Corex Corp., Gaithersburg, Md.) was added as an antifoam at a concentration of 0.003%. The culture was maintained for 16 to 18 h at 25°C, infused with 0.75 liters of air per min, and agitated at an impeller speed of 250 rpm. The culture supernatant fluids were recovered by centrifugation (16,000 × *g*; 20 min). Ammonium sulfate was dissolved in the culture supernatant fluids to a final concentration of 60%, and the precipitate was collected after 16 to 18 h by centrifugation (20,000 × *g*; 20 min) and dissolved in 36 ml of cold 0.1 M ammonium bicarbonate (pH 7.8). The solution was dialyzed for 16 to 18 h against 14 liters of 0.05 M ammonium bicarbonate and was

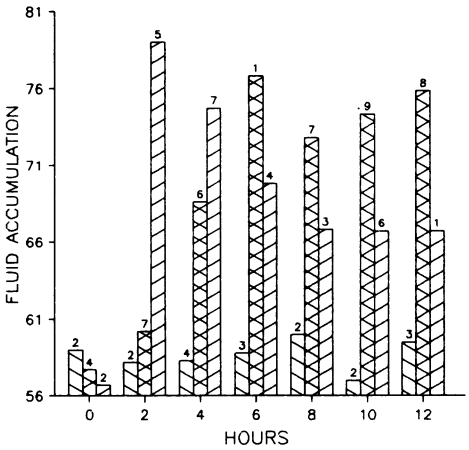

FIG. 2. Kinetics of FA ratios induced by *V. hollisae* ATCC 33564 and its partially purified enterotoxin preparation. Means of FA ratios for six mice are shown. Infant mice were starved, sealed, fed either 2×10^7 CFU of washed *V. hollisae* in 50 μl of PBS or 40,000 CHO units of toxin in 50 μl of PBS, and sacrificed at the indicated times, and the FA ratios were determined. At incubation times greater than 4 h, FA ratios induced by the bacteria were significantly higher than those induced by buffer ($P < 0.01$; paired Student *t* test). At incubation times 2, 3, 4, and 6 h postchallenge, FA ratios induced by the toxin were significantly higher than those induced by buffer ($P < 0.005$; paired Student *t* test). Mice challenged with either heat-killed (100°C for 30 min) *V. hollisae* or a heat-inactivated (56°C for 16 h) enterotoxin preparation did not cause fluid accumulation. Symbols: ▧, buffer; ▩, cells (2×10^7); ▨, toxin (40,000 CHO units). The numerals above the bars are the standard deviations.

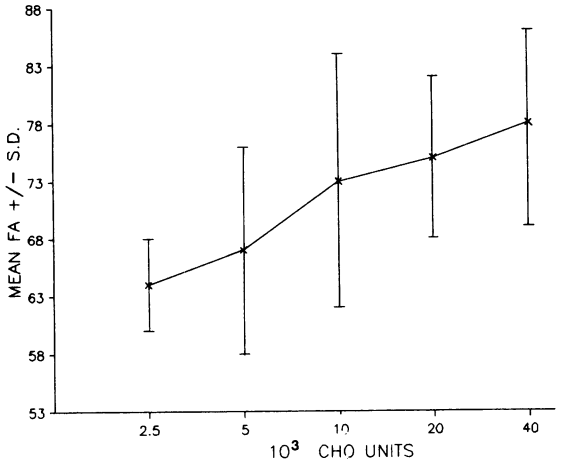

FIG. 3. Dose-response curve for the partially purified enterotoxin preparation of *V. hollisae* ATCC 33564. Mice were starved, sealed, fed the toxin in 50 μl of PBS, and sacrificed at 3 h postchallenge, and the FA ratios were determined. Control mice were fed 50 μl of PBS. At toxin doses greater than 10,000 CHO units, FA ratios induced by the toxin were significantly higher than those induced by buffer ($P < 0.01$; paired Student t test). Points represent the mean of FA ratios for six mice, and bars represent the standard deviation (SD).

lyophilized after centrifugation (25,000 × g; 20 min) to remove a small amount of insoluble residue. The lyophilized preparation was stored at −20°C.

Gel filtration. Gel filtration was performed by high-pressure liquid chromatography with a Protein Pak 125 column (0.78 by 30 cm; Waters Associates, Inc., Milford, Mass.) equilibrated with 0.1 M NaH_2PO_4–Na_2HPO_4 buffer, pH 7.0. A 10-mg amount of the lyophilized preparation dissolved in 100 μl of the buffer was applied to the column and eluted at a flow rate of 1 ml/min with the equilibrating buffer. Fractions were collected at 4°C, monitored for A_{280}, and assayed for CHO cell activity.

Hydrophobic interaction chromatography. A sample (1 ml) of the undialyzed ammonium sulfate-precipitated preparation was applied to a cooled (4°C) column (1.6 by 7.5 cm) of phenyl-Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) equilibrated with PBS (67 mM Na_2HPO_4–77 mM NaCl, pH 7). The column was washed (50 ml/h; ca. 3-ml fractions were collected) with ca. 6 bed volumes (90 ml) of equilibrating buffer, and the enterotoxin was eluted by washing the column (10 ml/h; ca. 0.5-ml fractions were collected) with 40% ethylene glycol in diluted PBS (6.7 mM Na_2HPO_4–7.7 mM NaCl, pH 7). The fractions were assayed for A_{280} and for CHO cell activity.

Isoelectric focusing. The partially purified enterotoxin preparation was fractionated by high-speed electrofocusing (19) in pH 3.5 to 10 and pH 2.5 to 5 sucrose density gradients formed at 15 W for 18 h with an LKB 8100-1 column (LKB Instruments, Inc., Gaithersburg, Md.). A 10-mg amount of the lyophilized enterotoxin preparation was fractionated in the pH 3.5 to 10 gradient, and 20 mg was used in the pH 2.5 to 5 gradient.

Molecular weight estimation by gel filtration. The apparent molecular weight of the enterotoxin was estimated by the gel filtration method of Andrews (1) with the Protein Pak 125 high-pressure liquid chromatography column as described above except that 2.5 mg of the sample was dissolved in 25 μl of buffer.

Inactivation studies. Samples of the lyophilized enterotoxin preparation were tested for sensitivity to heat (25, 27, 56, and 100°C for 30 min) and for sensitivity to inactivation at pH 4, 6, 7, 8, 9, and 10 (24 h at 4°C).

RESULTS AND DISCUSSION

Enterotoxin production. The addition of 0.5% NaCl to heart infusion broth (which contains ca. 0.5% NaCl) improved the yield of extracellular enterotoxin produced by *V.*

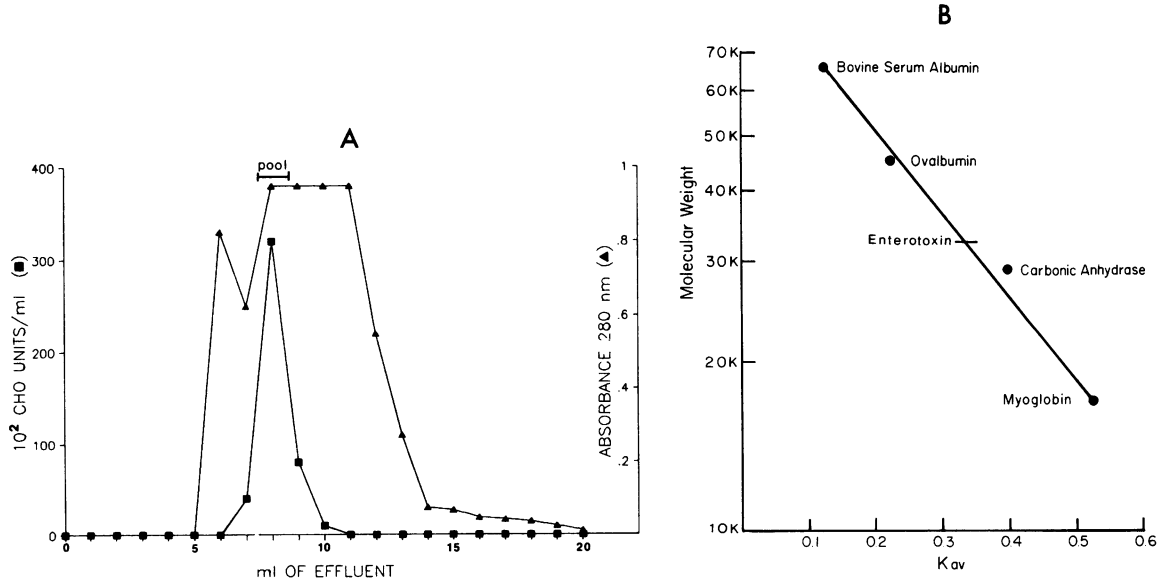

FIG. 4. Gel filtration of an enterotoxin preparation obtained from culture supernatant fluids of *V. hollisae* ATCC 33564 by sequential ammonium sulfate precipitation, dialysis, and lyophilization. (A) Elution pattern. Fractions were assayed for CHO cell activity and for A_{280}. The column void volume and bed volume were ca. 6 and 12 ml, respectively. (B) Estimation of apparent molecular weight of the enterotoxin by the method of Andrews (1).

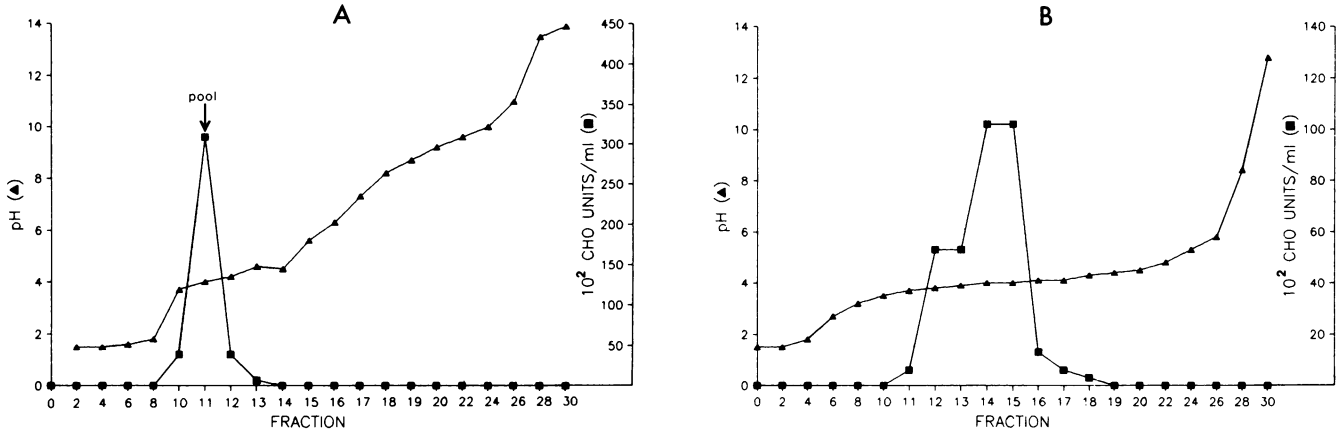

FIG. 5. Isoelectric focusing of an enterotoxin preparation obtained from culture supernatant fluids of *V. hollisae* ATCC 33564 by sequential ammonium sulfate precipitation, dialysis, and lyophilization. The pH of each fraction was determined at 4°C, and the fractions were assayed for CHO cell activity. (A) Results obtained with a pH 3.5 to 10 gradient. (B) Results obtained with a pH 2.5 to 5.0 gradient.

hollisae. Therefore, other media were adjusted to contain a final total concentration of 1% NaCl. Maximal amounts of extracellular CHO cell activity (32,000 CHO units per ml; one CHO unit is the reciprocal of the dilution that causes elongation of >50% of the CHO cells) were obtained from cultures grown in heart infusion broth and Columbia broth. Cultures from the other nine media had ca. 10 to 160 CHO units per ml.

Heart infusion broth was selected for the study of growth and enterotoxin production in a fermentor. Extracellular CHO cell activity was first detected after 6 h (late-exponential- to early-stationary-growth phase) of incubation at 25°C and increased to a maximal value of 32,000 CHO units per ml during the stationary-growth phase (Fig. 1). Incubation of the culture at 30°C (data not shown) did not improve the CHO cell activity but decreased the CFU after 14 h of incubation. There was no detectable protease activity against azocasein and no cytolytic activity against erythrocytes from mice, sheep, rabbits, humans, and chickens. The absence of detectable protease may be responsible for the stability of the CHO cell activity. Culture supernatant fluids were routinely harvested after a 16-h incubation period. About 80% of the CHO cell activity present in the culture supernatant fluids was recovered in the lyophilized ammonium sulfate-precipitated preparation. This preparation contained ca. 3.5×10^7 CHO units (20,000 CHO units per mg) and had a specific activity of ca. 100,000 CHO units per mg of protein.

Fluid accumulation induced by *V. hollisae*. The FA ratios induced by 2×10^7 CFU of *V. hollisae* increased up to 6 h and then remained stable (Fig. 2). Some mice did not survive beyond 12 h postchallenge. In addition, all mice challenged with 2×10^8 CFU died within 3 h postchallenge, and mice challenged with 2×10^6 CFU did not accumulate fluid. The median lethal dose of the *V. hollisae* by the peroral route was ca. 5×10^6 cells.

To determine whether *V. hollisae* proliferates in the intestinal tract, a mouse was challenged with 2×10^7 cells, and after a 12-h incubation period, the intestine and stomach were removed and homogenized in saline, and the CFU were estimated. The mouse exhibited an FA ratio of 0.071, and the intestine contained ca. 2×10^6 *V. hollisae* cells, indicating that there was no proliferation of the bacterium in the intestinal tract. In addition, the presence of ca. 8,000 to 32,000 CHO units in an extract prepared by adding 0.5 ml of PBS and macerating the intestine and stomach from each of the 12 other mice which were similarly inoculated suggested that the extracellular enterotoxin was produced in vivo.

Fluid accumulation induced by the enterotoxin. Fluid accumulation induced by 40,000 CHO units of the enterotoxin reached peak values 2 to 4 h postfeeding (Fig. 2). Comparison of FA values induced by the enterotoxin and by the cells (Fig. 2) by the paired Student *t* test indicates that there are no significant differences between the two ratios at incubation periods greater than 4 h. In addition, the FA ratios induced by the partially purified extracellular enterotoxin preparation were dose dependent (Fig. 3). However, only the values for the doses greater than 10,000 CHO units were different from those of buffer-fed controls by the paired Student *t* test at the <0.01 level. In comparison, a minimum dose of 0.5 μg of cholera toxin (2) equivalent to ca. 45,000 CHO units (unpublished results) was required to obtain a significantly increased FA ratio over controls in an infant mouse model.

Pooled fractions possessing CHO cell activity obtained by gel filtration (Fig. 4), isoelectric focusing (Fig. 5), and hydrophobic interaction chromatography (Fig. 6) of the

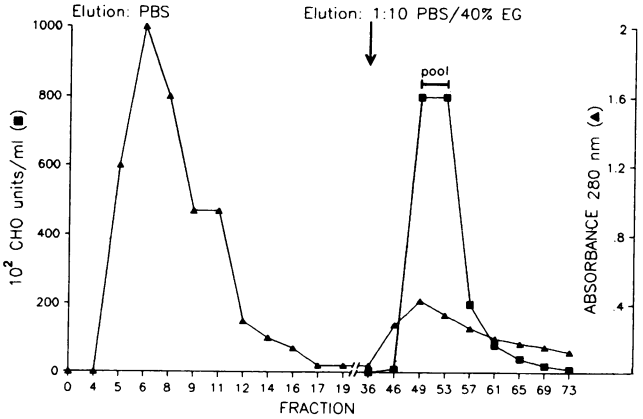

FIG. 6. Hydrophobic interaction chromatography of an enterotoxin preparation obtained from culture supernatant fluids of *V. hollisae* ATCC 33564 by ammonium sulfate precipitation. Fractions were assayed for CHO cell activity and for A_{280}. Fractions 1 to 36 were ca. 2.5 to 3.5 ml each, and fractions 37 to 73 were ca. 0.5 ml each. EG, Ethylene glycol.

TABLE 1. FA ratios induced by CHO cell-active pools of *V. hollisae* ATCC 33564

Sample[a]	FA ratios[b]
Gel filtration pool[c]	77 ± 7.2
Isoelectric focusing pool[c]	68 ± 3.6
Hydrophobic interaction chromatography pool[c]	68 ± 4.9
Control (PBS)	52 ± 1.5

[a] Mice were starved, sealed, fed either 40,000 CHO cell units of toxin in 50 μl of PBS or PBS alone, and sacrificed 3 h postchallenge, and the FA ratios were determined.

[b] FA ratios were determined for six mice per sample; values represent mean ± standard deviation. FA ratios induced by the toxin preparations were significantly higher than those induced by buffer ($P < 0.005$; paired Student t test).

[c] Pooled peak fractions were concentrated and washed on a Centricon-30 microconcentrator (Amicon Corp., Danvers, Mass.).

ammonium sulfate-precipitated preparation also elicited fluid accumulation in mice (Table 1). This observation suggests that the ability to elongate CHO cells and the ability to induce fluid accumulation in infant mice are most probably functions of the same product.

Estimation of molecular weight and isoelectric point. The molecular weight of the partially purified enterotoxin preparation was ca. 33,000 by gel filtration (Fig. 4). Isoelectric focusing of the enterotoxin preparation in a broad and shallow pH sucrose density gradient revealed only one peak of CHO cell activity that had an isoelectric point (pI) of ca. 4.0 (Fig. 5).

Inactivation studies. The enterotoxin lost 10 and 100% of its CHO cell activity when heated for 30 min at 56 and 100°C, respectively. The CHO cell activity was stable from pH 4 to 9 (24 h at 4°C).

Our finding that peroral administration of *V. hollisae* induced fluid accumulation in infant mice is similar to the observations made for O1 and non-O1 *V. cholerae*, *Vibrio fluvialis*, and *Escherichia coli* (2, 13, 16). Our data also suggest that the *V. hollisae* enterotoxin is similar both to the *V. cholerae* enterotoxin, the *E. coli* heat-labile enterotoxin, and the *V. parahaemolyticus* factor in its ability to elongate CHO cells (8, 10) and to the enterotoxins of *V. cholerae*, *E. coli*, and *Yersinia enterocolitica* (2, 5, 17) in its ability to elicit fluid accumulation in infant mice. However, it can be differentiated from both the *V. cholerae* enterotoxin, which has a pI of 6.6 and a molecular weight of 61,000 by gel filtration with Sephadex G-75 (7), and the *E. coli* heat-labile enterotoxin, which has a pI of 6.9 and a molecular weight of 102,00 by gel filtration with Sephadex G-150 (6). In addition, the inability of antisera raised against cholera toxin and against the CHO cell elongation factors of *V. fluvialis* and *V. parahaemolyticus* to neutralize the CHO cell activity of the enterotoxin (unpublished results) indicates that the *V. hollisae* extracellular enterotoxin is antigenically distinct from other CHO cell-elongating toxins. Studies are in progress in our laboratory to purify and characterize the enterotoxin so that its possible mode of action in the diarrheal disease caused by *V. hollisae* can be examined.

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